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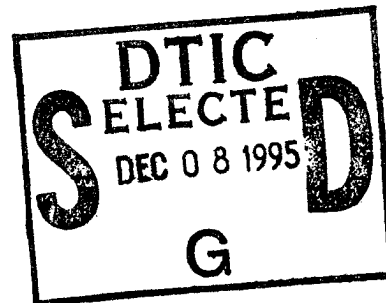
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**INT-3 ONCOGENE IN NORMAL AND
NEOPLASTIC BREAST DEVELOPMENT**
Annual Report 1995

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INTRODUCTION

I. NATURE OF THE PROBLEM

In the past several years, it has been shown that oncogenes contribute to the pathobiology of breast cancer. Mutational activation of the *int-3* oncogene, has been shown to contribute to experimental mammary gland tumorigenesis in mouse. Several human orthologues of the *int-3* gene have been implicated in human cancers. There is strong evidence that the int-3 protein regulates the cell fate decisions required for the morphogenesis and functional differentiation of the mouse mammary gland. Despite this evidence, work on the role of the *int-3* gene in breast cancer is still in its infancy. Extensive studies on other int-3 family members (lin-12/Notch) in organisms more tractable to genetic analysis such as *Drosophila* and *C. elegans*, demonstrates the evolutionary conservation of these proteins and their fundamental importance in cell fate decisions.

The proposed research will investigate the role of the int-3 protein in the normal physiology of breast development and study the biochemical properties that are important for int-3 transforming activity. This information will broaden our understanding about the events which control normal mammary gland development and how alterations in those events can lead to neoplastic growth of the mammary gland.

II. BACKGROUND

Mouse mammary tumor virus induces breast cancer in mouse by insertional mutagenesis. In tumors, viral integration can result in activation of the *int-3* gene by promoter insertion and results in expression of a truncated *int-3* gene product (2.3 kb RNA) (1). The nucleotide sequence of this truncated cDNA revealed homology with the Notch/lin-12 gene family (2). However the full length cDNA of the *int-3* gene has not been cloned. Several lines of evidence confirm a role for int-3 in mammary tumorigenesis. Transfection of a recombinant *int-3* genomic DNA fragment, encoding the truncated oncoprotein, into the HC11 mouse mammary epithelial cell line induces anchorage-independent growth in soft agar (2). Expression of this same genomic fragment *in vivo* as a transgene in a transgenic mouse strain is associated with arrest of normal mammary gland development and impaired differentiation (3), intraductal hyperplasia of mammary epithelium, and a high incidence of focal mammary tumors (adenocarcinomas) (4). It has also been reported that the normal *int-3* gene is endogenously expressed in the mouse mammary gland (5).

Int-3 is related to the Notch/lin-12 family of proteins. The Notch/lin-12 protein family currently consist of eleven members, Notch (*Drosophila*) (6), lin-12 and glp-1 (*C. Elegans*) (7, 8, 9), Xotch (*Xenopus*) (10), Notch 1, 2, 3 and int-3 (Mouse) (2, 11-15), Notch 1 and 2 (Rat) (16,17), NOTCH 1 and 2 (Human) (18, 19). These genes encode for transmembrane receptor proteins. The extracellular domain of Notch/lin-12 family members contains variable numbers of EGF (epidermal growth factor) like repeats and other cysteine rich repeats named lin-12/Notch repeats(26). The intracellular domain of all Notch/lin-12 family members contains several copies of a repeat sequence, named cdc10 or ankyrin repeat. The cdc10 repeats have recently been implicated as a protein-protein interaction domain. The intracellular domain of this family of proteins also contains a PEST sequence, a nuclear localization signal, and an opa repeat. PEST sequences are found in proteins which are rapidly degraded or may also represent potential phosphorylation sites. An opa repeat is a protein domain that is rich in glutamine and is commonly found in transactivating domains of transcription factors or transcription factor binding proteins (27). The Notch and lin-12 proteins are required for cell-cell interactions that play a pivotal role in cell-fate decisions. For instance, the mechanisms that control how a group of equivalent progenitor cells give rise to a group of cells each with their particular fate. The fundamental importance of these genes during development has been demonstrated by genetic analysis of lin-12, Notch and Xotch mutants (20-24). In the mouse, nul mutants of Notch 1 and 2 are lethal during embryonic development, although the exact cause for this premature death is not known (25 and unpublished data T. Gridley).

Genetic and molecular analysis have identified several proteins that participate in Notch signaling. *Drosophila Delta* (28) and *Serrate* (29) and *C. elegans Lag-2* (30) and *Apx-1* encode a family of structurally related ligands for the *Drosophila* Notch and *C.elegans* lin-12 and glp-1 receptors respectively. These ligands are transmembrane proteins, containing EGF-like domains and a cysteine rich DSL (Delta-Serrate-Lag-2) domain within the extracellular part of the protein. Recently, mouse homologs of these ligands have been cloned, *Jagged-1* (31) and *Dll-1* (32). These ligands have been demonstrated to regulate Notch receptor activity through cell-cell interactions. The products of three *Drosophila* genes, *deltex*, *disheveled* and *suppressor of hairless (Su(H))* have been shown to interact with the intracellular domain of Notch and may thus participate in the intracellular signaling pathway of Notch (33,34) Furthermore, genetic analysis has revealed similar phenotypes in certain *Deltex* and Notch mutants.

Deletion of the extracellular part of Notch, Xotch and lin-12 proteins results in a dominant gain of function mutation (20-22). The truncated gene product encoding for the intracellular part of the receptor exhibits constitutively activated protein function. The phenotype observed in this class

of mutants suggests that the truncated gene products delay cell determination and thereby increase the proportion of uncommitted stem cells, leading to a prolonged lifetime of the cell or to a greater number of descendants (20-22). By analogy to the function of other Notch/lin-12 family members in lower organisms, one can speculate that delay in differentiation and accumulation of pluripotent proliferative stem cells would result in a growth advantage, thereby increasing the probability for secondary oncogenic mutations. This model would propose that Notch proteins contribute to oncogenesis by stimulating stem cell growth and blocking differentiation.

Studies on the Notch protein in *Drosophila*, demonstrated that the intracellular part of the Notch protein is translocated to the nucleus when a truncated Notch protein (corresponding to the intracellular part of the protein) is expressed as a transgene in *Drosophila* embryos (20). Based on the hypermorphic effect of the deletion mutants, and on the presence of a nuclear translocation signal in the intracellular domain of the protein, a hypothetical model would be that ligand binding to the receptor would result in cleavage of the intracellular domain of the receptor and subsequent translocation to the nucleus, where it could interact with its substrate.

Notch/lin-12 gene family members have been implicated in human tumorigenesis. Alteration of NOTCH-1 (also named TAN-1) has been associated with a T lymphoblastic neoplasm (18). The mutation of the NOTCH-1 gene in T lymphoblastic lymphomas is caused by a translocation that results in expression of a truncated gene product. TAN-1 mutations are analogous to the *int-3* activating mutations as a result of MMTV insertion, as well as to the dominant gain of function mutations of Notch, lin-12 and Xotch. Furthermore, human NOTCH-1 and NOTCH-2 (also named hN) were found to be overexpressed in human cervical carcinomas (19).

III. PURPOSE

The *overall goal* of the work proposed here is to understand in molecular detail the function of the *int-3* protein in mammary epithelial cells and during mouse mammary gland development, with the *long term goal* of understanding the role of the *int-3* gene in mammary tumorigenesis.

IV. METHODS OF APPROACH

Our *general strategy* is to carry out an analysis of the normal *int-3* protein and the oncogenic *int-3* protein to determine how these proteins contribute to normal and neoplastic breast development by addressing the following *specific aims*:

Aim 1. *Compare the normal int-3 protein to activated int-3 oncoproteins.* The first step of this section will be to determine the primary sequence of the normal *int-3* protein and evaluate whether the normal *int-3* gene encodes a Notch-like transmembrane receptor. The second step will be to compare the biochemical properties, subcellular localization, and transforming activities of the normal *int-3* protein with that of truncated *int-3* oncoproteins. Our aim is to determine what structural alterations lead to oncogenic activation of *int-3* and to evaluate whether activated *int-3* proteins act at the cell surface and/or nucleus.

This aim has been divided into the following two *tasks*:

Task 1. *Characterize the full length int-3 cDNA* (Months 1-12).

We have cloned the truncated, oncogenic *int-3* gene and we have cloned and sequenced the full length *int-3* gene.

Task 2. *Study the production, processing, and subcellular localization of normal int-3 proteins and int-3 oncoproteins in mammary epithelial cells* (Months 3-18).

We have epitope-tagged *int-3* proteins, we have generated cell lines expressing the truncated *int-3* oncoprotein and we have evaluated the subcellular localization of *int-3* oncoproteins.

Aim 2. *Analysis of int-3 expression pattern in the murine mammary gland.* The fact that expression of the *int-3* oncogene in the mouse mammary gland has profound effects on the development of the gland suggests a role of the normal *int-3* gene products in mammary gland development. We will investigate whether the *int-3* gene is normally expressed in the mammary gland. More specifically, we will determine if *int-3* expression is found during distinct stages of mammary gland development and in distinct cell types of the mouse mammary gland. Molecular and immunohistochemical techniques will be used to evaluate where and when in the normal mammary gland the *int-3* gene is expressed.

We have made early progress on some tasks associated with this *specific aim*:

Task 5 (of proposal). *Analysis of the int-3 expression pattern in the murine mammary gland* (Months 12-48). We have initiated the generation of *int-3* specific antibodies and defined the expression pattern of this gene in mouse embryos and adult tissues.

BODY

I. EXPERIMENTAL METHODS/RESULTS

Characterization of the full length *int-3* cDNA.

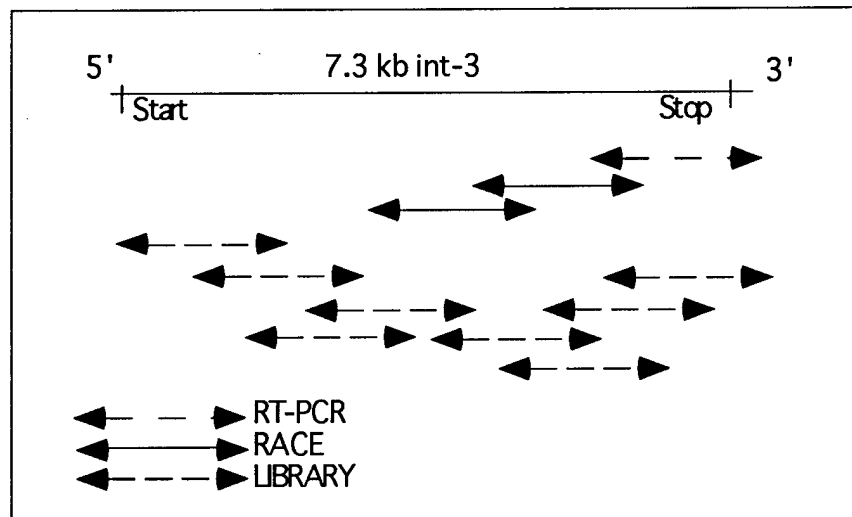
Int-3 has been classified in the *lin-12/Notch* protein family solely on the basis of its homology to the intracellular part of the *lin-12/Notch* family members. There has been no direct evidence that demonstrates that *int-3* is a transmembrane protein. By cloning the entire full length *int-3* cDNA, we have confirmed that the *int-3* gene encodes for a transmembrane protein, homologous to the *lin-12/Notch* family of transmembrane proteins.

A. Cloning truncated *int-3*. We have PCR amplified a fragment of the *int-3* gene that encodes for the intracellular part of the protein. Oligonucleotide primers were designed based on the published sequence (2). Mouse adult testis cDNA was used as template DNA in the PCR reaction. Appropriate sized fragments resulted from the PCR reaction and internal primers were used to confirm the identity of these PCR fragments. We have cloned the PCR products into a phagemid (pBluescript). Sequencing of these cloned fragment, revealed almost exact nucleotide identity to the published sequence (2). Some sequence differences result in a slightly different predicted amino acid sequence, for instance the coding frame predicts a transmembrane domain which was not reported in the published sequence of *int-3* (2).

B. Cloning of normal *int-3*. We have used the PCR based method of RACE (Rapid Amplification of cDNA Ends) (35,36) to clone sequences that are located 5' from the truncated *int-3* (the truncated *int-3* transcript is localized at the 3' end of the gene). To clone the *int-3* full length cDNA, we screened a mouse lung cDNA library. This choice of cDNA library was based on my findings of the mRNA expression analysis of *int-3* (task # 5). Initially, the probes that were used in this screening analysis were derived from the cloned truncated *int-3*, as well as from the clones obtained by RACE. Positive clones were purified and sequenced and used as probes in successive rounds of screening in order to obtain the full length cDNA of the *int-3* gene. The length of the *int-3* mRNA is approximately 7.3 kb based on our Northern blot analysis (task # 5). To date, I have cloned the 7.3 kb full length *int-3* cDNA (Fig 1). The cDNA contains a 400 bp 3' UTR (untranslated region) and a 500 bp 5' UTR, resulting in a 6.4 kb ORF (open reading frame). I have determined the initiating ATG based on Kozak rules, and based on its location 5' of a putative signal peptide domain. The *int-3* sequence has been analyzed for its homology with other *lin-12/Notch* family members, and has a high overall homology to the other known mouse *Notch* 1, 2

and 3 genes. It encodes for a transmembrane protein with a intracellular domain containing six ankyrin repeats, a transmembrane domain, and a extracellular domain containing three Notch/lin-12 repeats as well as up to more then twenty EGF-like repeats. We have also isolated *int-3* clones that are derived from differentially spliced *int-3* messages.

Fig 1: Schematic representation of *int-3* cloning



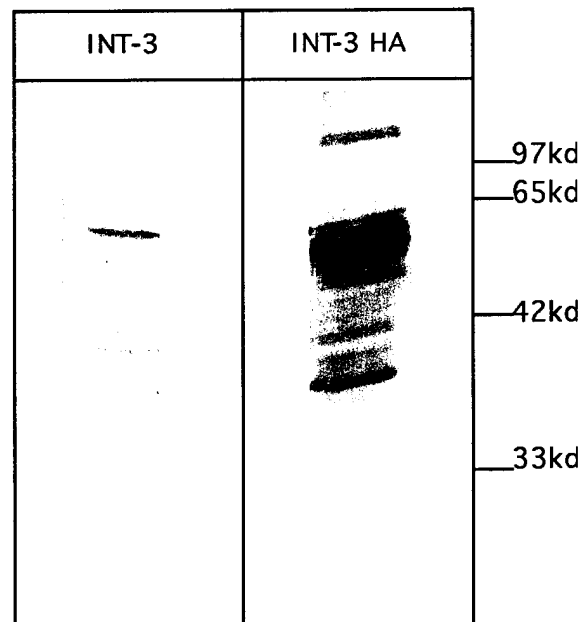
Determination of the biochemical properties of the *int-3* protein.

The *int-3* gene was discovered in mouse mammary tumors, induced by MMTV infection. Insertional mutagenesis by MMTV results in expression of a truncated *int-3* protein product that is able to transform mammary epithelium cells both in vivo and in vitro. We will analyze the biochemical characteristics of the *int-3* protein using biochemical, biological and molecular strategies. We will compare the biochemical properties, subcellular localization and transforming activities of the normal *int-3* protein with that of truncated *int-3* oncoproteins.

A. Epitope tagging of truncated *int-3*. Since antibodies are not immediately available against the *int-3* protein (task # 5), we have added an epitope to the coding region of truncated *int-3*(37). The epitope we have chosen to add to the *int-3* protein is derived from the influenza HemAgglutinin (HA) protein and is recognized by a monoclonal antibody 12CA5 (38). *Int-3* cDNA was cloned into phagemid vectors that contain the sequence encoding the HA epitope situated downstream. Single strands were generated from the phagemid and used in a site directed mutagenesis protocol with an oligonucleotide designed to loop-out the sequence between the last

int-3 codon and the first HA codon, to create in frame a cDNA encoding the *int-3*/HA fusion protein. The HA epitope was fused to the carboxy terminus of the *int-3* protein. This method allowed me to detect ectopically expressed *int-3*/HA fusion protein in transfection experiments, using anti HA antibodies in Immunoblot analysis. Cell lysates were prepared from *int-3* expressing cell lines, fractionated by SDS gel preparation, and transferred to nitrocellulose. This membrane was blotted with anti HA antibodies and the truncated *int-3* protein was detected by ECL (enhanced chemiluminescence) followed by autoradiography (Fig 2).

Fig 2: Western blot analysis on total cell lysates of transient transfected BOSC cells



B. Construction of truncated *int-3* expressing cell lines. An *int-3*/HA fusion construct was subcloned into a murine leukemia virus (MLV) based vectors, denoted LNCX vectors (39). These vectors utilize the cytomegalovirus immediate early promoter/enhancer to drive expression of the *int-3* gene and the retroviral LTR to drive expression of the *neo* gene, which confers resistance to the drug G418. We have also generated several control retroviral vectors, such as vectors not containing *int-3*, vectors containing an unrelated HA tagged protein, and vectors containing *int-3* not HA tagged. This last control vector will allowed us to investigate whether HA tagging (at the carboxy terminus) can interfere with the transforming activity of *int-3*. In order to evaluate the biochemical properties and transforming potential of *int-3*, mammary epithelial cell lines programmed to express *int-3* were generated by infection with retroviral expression vectors. High-titer helper free retroviral stocks were generated using the BOSC23 ecotropic virus packaging cell line (40), media was collected and used to infect mammary epithelial cell lines and fibroblast cell

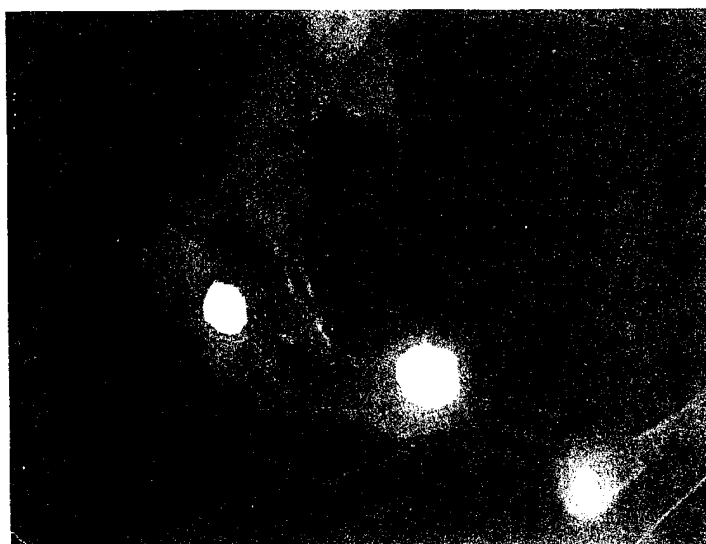
lines. The mammary epithelial cell lines that will be used, are C57MG(41), HC11(42), TAC-2 (43) and MCF7(44). HC11 will be used because it has been reported that this cell line can be transformed by truncated int-3 protein (2). In addition we will infect other established mammary epithelial as well as non-epithelial cell lines. Once these cell lines are established, the biochemical properties and transforming activity of int-3 will be investigated.

Determine the subcellular localization of int-3 proteins.

The biochemical properties of int-3 will be investigated once the full length cDNA has been expressed in mammary epithelial cells. Initially, we have determined the intracellular localization of truncated int-3.

From studies on the Notch protein in *Drosophila*, there is evidence that truncated Notch protein (corresponding to the intracellular part of the protein) is translocated to the nucleus when this protein is expressed as a transgene in *Drosophila* embryos. Because the subcellular localization of truncated int-3 might give a clue as to how wild type int-3 functions, we have examined the intracellular localization of truncated int-3 in transiently transfected cells (293T and HeLa cells). We have investigated the intracellular localization by indirect immunofluorescence (45) using the anti-HA antibodies, and have found nuclear localization of truncated int-3 (Fig 3). Several deletion mutants have been generated in order to determine which part of the truncated int-3 protein is responsible for its nuclear translocation. These initial studies pointed out two domains that may contain a nuclear translocation signal, one N-term and one C-term of the ankyrin repeats. Interestingly, deletion of the ankyrin repeats alone did not affect the nuclear localization. Preliminary data suggest that a protein consisting solely of the int-3 ankyrin repeats resides in the cytoplasm.

Fig 3: Nuclear localization of int-3 in transiently overexpressed 293T cells



Analysis of the int-3 expression pattern.

We will analyze the expression pattern of the int-3 protein as well as the *int-3* mRNA. Such analysis will give further insights into the function of the int-3 protein. First, we will investigate whether int-3 is expressed in the mammary gland and whether this expression is developmentally regulated. Second, we will study the expression pattern of int-3 in different mouse tissues, to define tissue specific expression or ubiquitous expression of int-3.

A. Generating int-3 specific antibodies. To study the endogenous expression pattern of the int-3 protein (task # 5), polyclonal antibodies against GST-int-3 fusion proteins are being generated. The intracellular part of the int-3 protein was fused in frame to GST and purified over a glutathionine column. Purified fusion proteins have been injected into two rabbits. The specificity of the polyclonal antibodies will be analyzed as soon as bleeds are available. We expect to obtain antiserum as soon as 10/95.

B. Expression analysis of int-3. Int-3 protein expression will be studied once the int-3 specific antibodies (see task # 5) for Western blot analysis are available. Immuno-histochemistry using those int-3 specific antibodies will be used to determine the specific localization of int-3 protein in tissue section and define the specifically the cell types expressing int-3 protein. *Int-3* mRNA expression has been studied by Northern blot analysis using probes derived from the 3' UTR of *int-3* (see task # 1). The *int-3* gene encodes for a 7.3kb transcript that is expressed in lung, heart, and kidney in adult tissues. Several shorter *int-3* transcripts were observed in adult testis, and appear to consist of alternatively spliced transcripts. The *int-3* transcript is expressed at all stages of mouse development (day 6.5 to 15.5). In situ hybridization (using the same probe as in the Northern blot analysis) was performed to determine the source of *int-3* expression during mouse development, and revealed endothelial specific expression. In situ hybridization on adult lung tissue is currently performed to determine the cell type in lung that expresses *int-3*.

II. GOALS OF THE RESEARCH

The goal of the proposed research is to investigate the role of the *int-3* protein in the normal physiology of breast development and study the biochemical properties that are important for *int-3* transforming activity. The data presented in this annual report represents results of experiments outlined in *specific aim 1* and *specific aim 3* of the research proposal. We have completed all of *task 1* of *specific aim 1* (Months 1-12) by cloning both the truncated *int-3* oncogene and a full length, 7.3 kb cDNA encoding normal *int-3*. We have completed approximately half of *task 2* of *specific aim 1* (Months 3-18) by epitope tagging *int-3* proteins, by generating retroviral vectors for expressing *int-3* proteins and by defining the subcellular localization of the *int-3* oncoprotein. In addition, we have made early progress towards analyzing the *int-3* expression pattern in the murine mammary gland, *task 5-specific aim 3* (Months 12-48) of research proposal (*aim 2* of this annual report). We have already purified bacterially produced *int-3* proteins, injected these proteins into rabbits, and are commencing to determine whether the rabbits are producing *int-3* specific antibodies. We have also defined the embryonic expression pattern of *int-3* and defined adult tissues where *int-3* is expressed. We will next look specifically in the murine mammary gland for *int-3* expression.

CONCLUSIONS

In conclusion, we have cloned both the truncated, oncogenic *int-3* gene and have cloned a full length cDNA of approximately 7.3 kb which encodes the normal int-3 protein. The deduced amino acid sequence of the normal int-3 protein displays significant homology to other Notch family members. The extracellular domain is characterized by the presence of a putative signal peptide followed by a series of EGF-repeats and Lin12/Notch repeats. The truncated *int-3* gene has been cloned and epitope tagged. Cell lines have been generated that are programmed to express the truncated int-3 protein. The truncated int-3 protein expressed in epithelial cells was detected by Western blot and immunofluorescence analysis. We have found that the truncated int-3 protein is localized to the nucleus, suggesting that signal transduction may be mediated by the nuclear transport of the intracellular part of the int-3 cell surface receptors. Now that the full length *int-3* gene has been cloned, these experiments will be expanded to biological activity and biochemical properties of the normal int-3 protein.

The expression analysis of the *int-3* mRNA was analyzed in adult tissues as well as during mouse development. The *int-3* transcript consists of a 7.3 kb mRNA species found in lung, heart, kidney, and mouse embryos. Embryonic expression appears to be restricted to endothelial tissue. Future work will focus specifically on the expression of int-3 in the murine mammary gland.

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